Detailed Online Methods for Ms. #5126-R1

RBP-null Mice

Production of RBP mutant mice was previously described by Quadro et al.[1] This mutant line, which had been maintained on a mixed genetic background of 129/Sv and C57BL/6, was re-derived after transfer to the Medical College of Wisconsin Animal Resource Center for placement in its barrier facility. For re-derivation, heterozygous blastocysts obtained by mating RBP-null males with WT CD-1 females were implanted into pseudopregnant CD-1 WT females. Heterozygous progeny were back-crossed with WT C57BL/6 mice, and heterozygous progeny (RBP+/-) of these matings were bred to generate RBP-null offspring, verified by Southern blotting as described in Quadro et al.[1] WT progeny of RBP-/+ matings were used to establish a line of normal control animals having an identical background to the RBP-null mice. All mice used in this study were fed *ad libitum* Purina rodent chow #5010, which contains 44 IU vitamin A (acetate) per gram diet and 4.5 ppm carotene.

Collection & Cryo-Preservation of Embryos

In all instances, harvested embryos were carefully staged by enumerating somite pairs. Embryos were immediately fixed by cryo-preservation,[2] a technique that optimally maintains antigenicity while preserving extracellular matrix structures. Briefly, embryos were collected and washed in phosphate buffered saline (PBS), placed on copper EM grids or 'sleds' and fixed by plunging into 2-methylbutane cooled with liquid nitrogen. After 15-30 seconds in 2-methylbutane the embryos were transferred to vials of frozen N,N-Dimethylformamide (DMF) which were placed in a glass jar, covered with liquid nitrogen and stored at -80^{o}C. After three days vials were transferred to -20^{o}C for 8 hours and placed at 4 ^{o}C overnight. The next day, embryos were equilibrated to room temperature, stained with toluidine blue in 100% ethanol for three minutes and embedded in Paraplast according to standard procedures. Microscopic sections were removed at 7 μm thickness.

Histologic & Immunohistochemical Staining

Sections were de-paraffinized according to standard procedures. Conventional histologic staining utilized hematoxylin and eosin. For immunohistochemistry, embryonic sections were blocked in a humid chamber at 4^{o} C overnight in PBS containing 5% BSA/0.05% Triton X-100; this blocking solution was also used as the diluent for primary and secondary antibodies.

Sections were covered with appropriately diluted (see below) primary antibody and incubated overnight at 4° C; for controls the primary antibody was omitted. Primary antibodies included rabbit anti-fibronectin (1:200; Chemicon #AB2033) and mouse anti-α-sarcomeric actin (1:800; Sigma, #A-2172). The next day, sections were thoroughly washed with blocking solution and secondary antibody was applied for 60 minutes at RT. The secondary antibody for detection of fibronectin antigen-antibody complexes was fluorescein-conjugated goat anti-rabbit IgG (ICN, #55662) diluted 1:100; for detection of α-sarcomeric actin complexes fluorescein-conjugated goat anti-mouse IgM (1:200; ICN, #55498) was used. To counter-stain nuclei after immunostaining, 100 ng/ml 4', 6 diamidino-2-phenylindole dihydrochloride (DAPI) in 1x PBS was applied for 15 minutes at room temperature. After staining, sections were thoroughly washed with blocking solution, rinsed with PBS, mounted with coverslips using Fluoromount-G (Southern Biotechnology Assoc.) and observed under epifluorescent illumination.

Digital Image Analysis

The RBP-null phenotype was characterized in terms of whole heart volume, myocardial wall volume and cushion area via quantitative analysis using Metamorph Video Imaging System® imaging of sections that were immunostained with sarcomeric α-actin. Because this antibody intensely and specifically stains myocardium, imaging of this myocardial area could be discretely captured for analysis. All Metamorph analyses were performed on sections from E9.5 embryos sections immunostained with α-sarcomeric actin.

Electron Microscopy

Electron microscopic analysis was performed on hearts from E9.5 and E10.5 embryos; at each stage three WT and three RBP-null hearts were evaluated. Dissected hearts were rinsed with PBS and fixed in 2.5% glutaraldehyde for 2 hours at room temperature. Following fixation hearts were embedded in epoxy resin, sectioned and imaged on a Hitachi 600 electron microscope at the Medical College of Wisconsin Facility for Electron Microscopy. Sections were evaluated at 8,000x and 20,000x magnification, in blind, to assess extent of cytodifferentiation based on enumerated nascent Z-discs and adherens junction structures. This analysis was confined to 'compact layer' myocytes that comprise the first layer of sub-epicardial myocardium. In each heart 22-26 cells were examined. Standard error and t-test analyses were applied to determine statistical significance.

BrdU Analysis

5'-bromo-2'-deoxyuridine (BrdU; 10 mg/ml) was prepared in 0.9% NaCl and 0.1mg BrdU/gram body weight was injected into the peritoneal cavity of pregnant 10.5 dpc mice. Four hours later, embryos were processed for immunohistochemistry as described above. After placing sections on slides, bromine moieties in DNA were 'unmasked' by incubation in 4N HCL for 20 minutes at RT followed by thorough washing in PBS. Sections were blocked with 5% BSA/0.05% Triton X-100 in PBS, overnight at 4^{o}C. Primary mouse anti-BrdU antibody (Becton Dickson #347580) was diluted 1:250 in blocking solution and incubated on sections for 1 hour at RT. After thorough washing with blocking solution the secondary antibody, fluorescein-conjugated goat anti-mouse IgG (ICN, #55496) diluted 1:500, was applied to the sections for 90 minutes at room temperature. After thorough washing nuclei were stained with propidium iodide for 2 minutes at a concentration of 1 μg/ml in water. Five hearts each from WT and RBP-null embryos were subjected to analysis of BrdU incorporation. From each heart, random sections containing an aggregate of at least 1,000 ventricular cardiac myocytes were enumerated to determine the percentage of BrdU-positive cells, as calculated from the ratio of nuclei exhibiting BrdU to total nuclei stained with propidium iodide. In addition to enumerating total myocardial myocytes, separate counts of trabecular and sub-epicardial myocyte subsets were performed. To confirm results these enumerations were performed in blind by two investigators who recorded essentially identical values. Statistical significance was determined by subjecting data to standard error and t-test analyses.

Western Blot Analysis

Embryonic mouse hearts and livers were collected, rinsed three times in 1x PBS, flash-frozen in liquid nitrogen and stored at -80^{o}C. Immediately prior to electrophoresis, whole hearts or livers were rapidly dissolved in a minimal volume (10-100 μl depending on embryonic stage) of ice-cold gel loading buffer (6.0 M urea, 50 mM Tris [pH 6.8], 0.2% SDS, 100 mM DTT, 0.1% bromophenol blue) containing 1x protease inhibitor cocktail (Roche #186153). Dissolved samples were heated at 100^{o}C for 4 minutes and immediately loaded onto a 7.5% acrylamide/SDS gel for electrophoretic separation. To compare Fn quantity in WT and RBP-null hearts at each stage, total protein from whole individual hearts (E9.0), or identical fractions of whole hearts (E9.5 - E12.5), was loaded in adjacent lanes. After electrophoresis at 150 Volts for 1 hour, protein was electro-blotted onto a nitrocellulose membrane (0.2 μm pore size, Bio-Rad #162-0146) at 100 V for 2 hours using transfer buffer containing 50 mM Tris, 380 mM

glycine, 20% methanol, and 0.1% SDS. After transfer, membranes were rinsed with TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.05% Tween-20) for 5 minutes, followed by incubation overnight at 4 ^{o}C in blocking buffer (5% non-fat dry milk in TBST). After blocking, membranes were incubated for 1 hour at RT with fibronectin primary antibody (Chemicon #AB2033) diluted 1:2000 in blocking buffer containing 1% goat serum). Membranes were rinsed 3X with blocking buffer and incubated with goat anti-rabbit IgG (H+L)-HRP conjugate secondary antibody (Bio-Rad #170-6515) diluted 1:3,000 in blocking buffer plus 1% goat serum for 1 hour at RT. Blots were rinsed 4X with TBST (without non-fat dry milk) followed by incubation with ECL reagent (Amersham Bioscience #RPN2106) according to the manufacturer's recommendations. Fibronectin signal was detected by exposing blots to Hyperfilm ECL (Amersham-Pharmacia Biotech #RPN3114k) for 5-30 seconds.

Quantitation of Fibronectin Protein Expression

Images from immunostained western blots were processed on an AlphaImager^{TM} 2000 system utilizing AlphaEase^{TM} software to calculate the area and signal intensity of fibronectin bands in stage-matched WT and RBP-null sample pairs. Since the RBP-null samples always displayed a stronger Fn signal, the RBP-null:WT ratio was calculated for each pair and utilized to determine the percentage increase of Fn expression in RBP-null hearts and liver. At each stage, calculations were based on samples processed from the following numbers: E9.0, 14 WT and 11 RBP-null hearts; E9.5, 8 WT and 10 RBP-null hearts; E11.5, 2 WT and 2 RBP-null hearts; E12.5, 1 WT and 1 RBP-null heart; E12.5 liver, 1 WT and 1 RBP-null liver.

Northern Hybridization

Embryonic heart tissue was rinsed 3 times with 1X PBS (nuclease-free), flash-frozen in liquid nitrogen and stored at -80^{o}C. RNA was isolated from one WT E10.5 whole embryo, one RBP-null E10.5 whole embryo, one pool of 53 WT E10.5 hearts, and one pool of 55 RBP-null E10.5 hearts. Total RNA was purified using the RNeasy Mini kit (Qiagen), according to the alternate proteinase K protocol, which is designed to increase RNA yield from contractile protein-rich tissue. RNA was spectrophotometrically quantitated at A_{260} and 1 μg total RNA was loaded per lane on a 1% agarose/formaldehyde gel. Electrophoretically separated RNA transcripts were transferred by capillary action to a BrightStar-Plus nylon membrane (Ambion #10100) and crosslinked to the membrane with ultraviolet light. The mouse Fn cDNA used for generating an antisense riboprobe was created by sub-cloning a 3.1 kbp Kpn I-Sac I fragment from IMAGE clone #3497891 (GenBank Accession #BG519292) into pBluescript-KS. After digestion with

XhoI, activation of the T3 promoter using the MAXIscript *in vitro* transcription kit (Ambion) generated a 665 nt antisense riboprobe labeled with [α-^{32}P]-UTP. Riboprobe was purified using the RNeasy kit and 5 x10^6 cpm per ml were diluted in NorthernMax hybridization solution (Ambion). Following overnight hybridization at 65 ^{o}C, blots were washed, exposed to BioMax film (Kodak #8294985) for 24-72 hours and developed to detect duplexed transcripts.

Real-Time Polymerase Chain Reaction

First-strand cDNA was reverse-transcribed from 2.5 μg of WT or RBP-null RNA using random hexamers (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen); reverse transcriptase was omitted from some reactions to control for genomic DNA contamination. In the amplification reaction, an aliquot of this cDNA was incubated with 10 pmol of each primer oligodeoxynucleotide and 2 μl Lightcycler Master SYBR Green I mixture (Roche Molecular Biochemicals). Fluorescent product at the end of each amplification cycle was quantified using the LightCycler (Roche Molecular Biochemicals), and, at the conclusion of the last amplification round, a melting curve analysis was performed on all samples to insure that only specific amplification occurred. Each series of amplification reactions was accompanied by a standard curve, which was comprised of parallel amplification reactions using cDNA generated from normal mouse total RNA, and serially diluted to span the range of values obtained from the unknown samples. Primers for Fn (GenBank Accession #AA900988) detection were 5'-GTGTTTGGACACAGCCACAG-3' (forward) and 5'-TGTGATTTGGTCTGGGATCA-3' (reverse). Primers for β-actin (GenBank Accession #NM031144) detection were 5'-AGGCATCCTGACCCTGAAGTAC-3' (forward) and 5'-GAGGCATACAGGGACAACACAG-3' (reverse). Primers were designed to amplify products of 200 - 300 bp.

In Situ Hybridization

Transcripts encoding RBP and Fn were detected in somite-matched embryos that were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned at 7 μm. Hybridizations were performed as described[3] except that the proteinase K (20 μg/ml) digestion time was only three minutes for E9.0 - E11.5 embryos. The Fn antisense riboprobe was identical to that used for northern analysis. Full-length RBP antisense riboprobe was transcribed from IMAGE Consortium clone #303723 (GenBank Accession #AI605545). Sections were hybridized with sense or antisense riboprobes labeled with [α-^{35}S]UTP that were pre-digested under alkaline conditions to an average size of 750 nt. Exposure times were 3 days for Fn mRNA and 7 days

for RBP mRNA. Following development, tissues were counterstained with propidium iodide and photographed under fluorescence and dark-field imaging. Whole mount *in situ* hybridization was performed on E9.5 and E10.5 embryos using established protocols;[4] the MLC-2v and MLC-2a cDNAs were kind gifts from Dr. Kenneth R. Chien (UCSD).[5] In all studies, hybridization with sense cRNA served as the negative control and in no instance was a hybridization signal detected in those samples.

References

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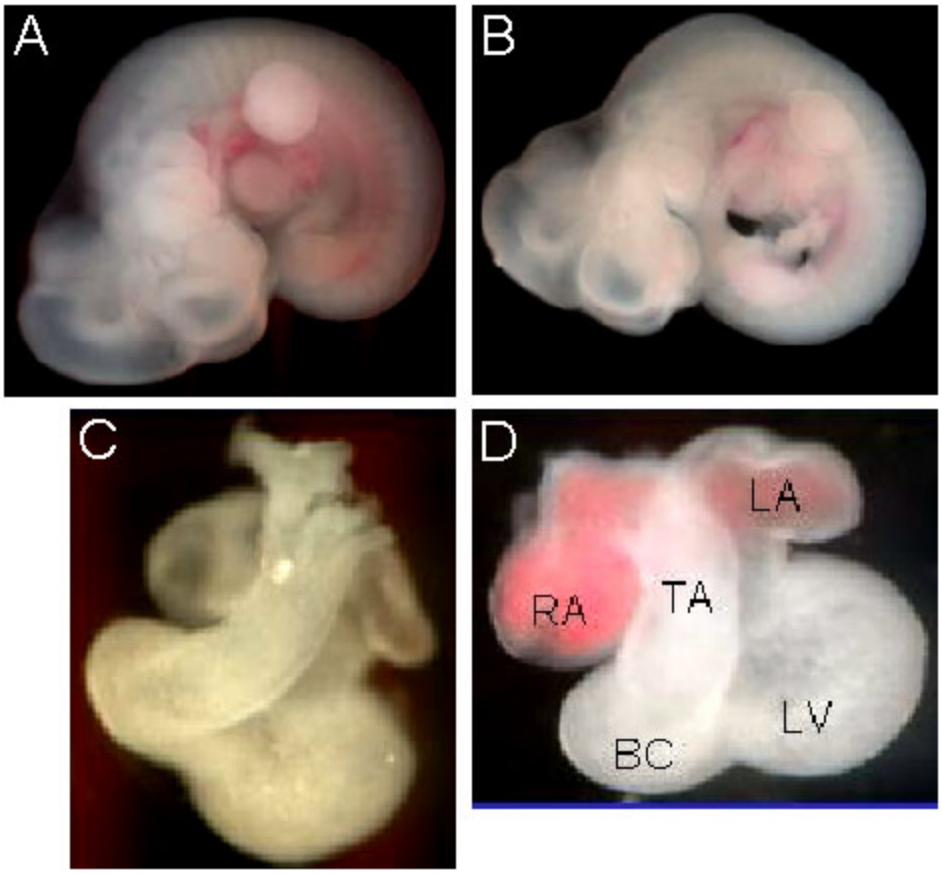
Figure Legends for Ms. #5126-R1

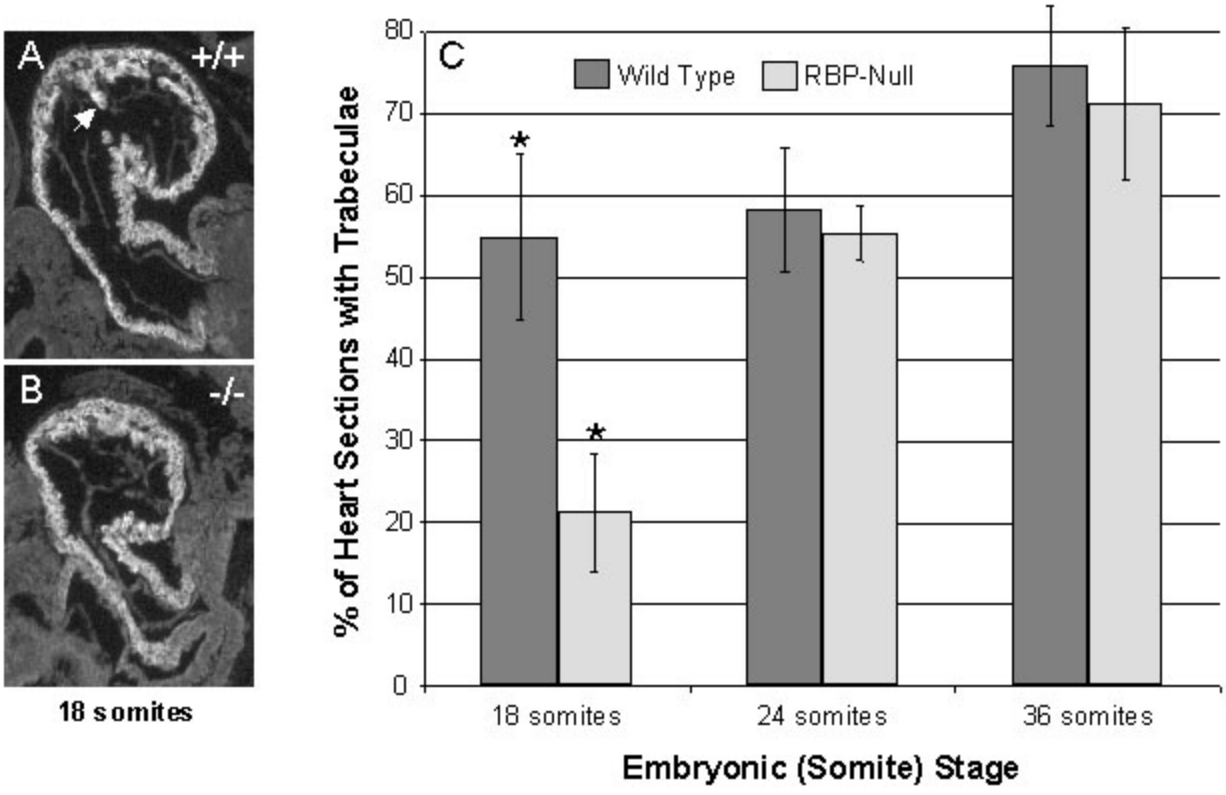
Online Figure 1. Gross Morphology of WT and RBP-null Embryos and Embryonic Hearts at E10.5. Panels A and B depict whole embryos; panels C and D depict embryonic hearts. WTs = A and C; RBP-nulls = B and D. No discernable differences in morphology due to RBP gene deletion are apparent. In D: RA = right atrium, LA = left atrium, TA = truncus arteriosus, BC = bulbus cordis, LV = left ventricle.

Online Figure 2. Decreased Trabeculation in Myocardium of 18 Somite Stage (E9.0) RBP-Null Embryos. Sections from somite stage-matched WT (A) and RBP-null (B) embryos were immuno-stained with anti-sarcomeric α-actin antibody as described in *Methods*. Morphological quantitation (panel C) revealed that trabeculation in RBP-null hearts was >50% less than WT at somite stage 18 (E9.0; *P<= 0.027). For these assessments, a section was considered to be trabeculated if it possessed any finger-like projections into the lumen of the heart that were at least as long as the myocardial wall was wide. For these assessments, 3 pairs of 18 somite embryos, 4 pairs of 24 somite embryos, and 3 pairs of 36 somite embryos were evaluated. The white arrow in panel A points to a trabeculum.

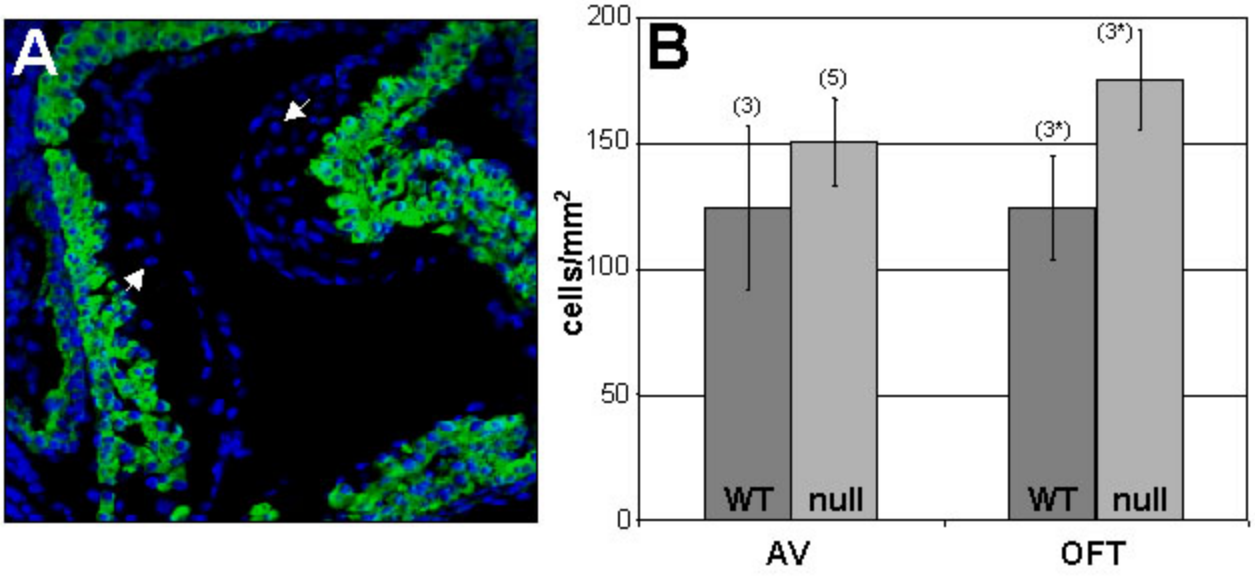
Online Figure 3. Increased Cells in Outflow Tract Cushions of E9.5 RBP-null Hearts. Panel A: Sections of WT and RBP-null E9.5 hearts were immunostained with anti-sarcomeric actin antibody (green) and nuclei were counterstained with propidium iodide (blue). Nuclei of mesenchymal cells that were clearly within circumscribed areas corresponding to the atrio-ventricular (AV) and outflow tract (OFT) endocardial cushions (shown in A) were enumerated; the inclusion of endocardial cells in these enumerations was assiduously avoided. Within each heart, cells in all sections through the AV or OFT cushions were enumerated. Quantitative results are shown in panel B, indicating that the OFT, but not AV, cushions in RBP-null hearts contained significantly increased numbers of mesenchymal cells. Vertical bars = standard errors of the mean; numbers in parentheses indicate the numbers of mice that were analyzed. Statistically significant differences between means was determined from the Student t-Test; *P<= 0.018. Arrows denote area of endocardial cushion.



Online Figure 1



Online Figure 2



Online Figure 3